Chapter III

MATERIALS AND METHODS

3.1. Field survey

Survey was conducted in different places of Assam for collecting the information on plants used by the traditional medicinal practitioners. The purpose of this study was to assess the traditional medicinal plants knowledge for treatment of various diseases with special regard to pneumonia. Ethnomedicinal information was collected through personal communication with senior tribal and local people of the villages. Communication was carried out in local language.

3.1.1. Survey site

Mayang, kajali chauk of Morigaon district (latitude: 26.15° N and longitude: 92°E) that occupies an area of 1,704 square kilometres (658 sq mi). The North part of the district is surrounded by the mighty Brahmaputra and the South part by Karbi Anglong district. Nagaon District is on the East of Morigaon and Kamrup District on the West. The district has majority of alluvial plain land area. Morigaon district harbours many waterways, rivers and marshes. Mustard, wheat, jute, paddy, and vegetables are the chief crops grown in this place.
We got information about various plants that were used locally for treatment of pneumonia. Mayang is famous for folk medicine since the ancient time. Based on information of herbal healers we collected few plants which were used in pneumonia treatment, namely titaful (*Phlogocanthus thyrsiflorus*), pipali (*Piper longum*), and Bandar kekua (*Mucuna prurita*).

Survey site also includes Rangia (latitude: 26.28° N and longitude: 91.38°E) and Baksa district (latitude: 26.67° N and longitude: 91.35°E). Baksa district has a population density of 475 inhabitants per square kilometre (1,230/sq mi). The district is bounded by major part of the world famous Manas National Park. Survey area in this district include Tamulpur, Kumarikata, Patkajuli, Sesapani, Garhali and few other places. The traditional healers and local people of this area informed that Tamulpur area is highly pneumonia affected and people of that place generally use *Mucuna prurita* seeds for treating pneumonia. *Phlogocanthus thyrsiflorus* is used traditionally for treatment of pneumonia, cough and fever in this area. Places like Naokota, Jabarangpara, Tanganmara and Chawabari of Baksa district, Assam were also surveyed where we got information about various plants used locally for treatment of pneumonia and other respiratory tract diseases.

Different places of Kamrup district (situated between 25.43 and 26.51 North Latitude and between 90.36 & 92.12 East Longitude) were also surveyed and plants were collected on the basis of ethnomedicinal information.
3.2. Plant collection and extract preparation

Plants were collected in field in flowering state as far as possible. Collected plant parts were shade dried and then ground to fine powders. Powders were stored in sealed plastic bags until use.

For extract preparation the ground samples were weighed and transferred to thimble of a Soxhlet. Then a particular amount of solvent was poured over the powder. Extraction of the plant samples was carried out with a series of solvents viz. petroleum ether (PE), chloroform (CHF), methanol (MT) using Soxhlet apparatus and aqueous extract (AQ) was
prepared by soaking the powdered samples in water for 48 h. Concentrated extracts were obtained by evaporating the solvent using rotary vacuum evaporator (Harborne 1998). Filtered water extract was dried by using lyophiliser. Extracts were stored in 15ml glass vial and kept at 4°C until use.

3.3. Microbial strains

Four reference strains of human pathogenic bacteria were used in this study including two gram positive viz. *Staphylococcus aureus* (96) and *Streptococcus pneumoniae* (655) and two gram negative viz. *E. coli* (443) and *Pseudomonas aeruginosa* (424). Bacterial strains were procured from the Institute of Microbial Technology (IMTECH), Chandigarh. The stock cultures were maintained in nutrient agar (NA) slant at 4°C and sub-cultured every two months.

3.4. Microbial media preparation

For preparation of slant 1.5% of nutrient agar (Himedia RM026) was added to water and then boiled and stirred until all the agar is melted and distributed throughout the media. About 5ml of the melted media was transferred to the test tubes and the tubes were then closed with caps and then sterilized at 121°C for 15 min. The test tubes were kept slanted till the media got solidified. For preparation of broth cultures one week old bacterial plates were used. Plates were prepared by pouring 30 ml of melted agar in sterile disposable petri plates (Himedia, 90x15 mm) and then allowed to cool. For preparation of active cultures one loopful of each bacterial strain was inoculated in 10ml of sterilized nutrient broth (NB) and incubated for 18hrs at 37°C.
3.5. Microbial assay

Disc diffusion method was used for preliminary antibacterial screening (CLSI 2009). Muller Hinton Agar media (MHA, Himedia M1084) was used for screening. 100 µl of fresh bacterial culture was placed over the dried agar plates and then spread with the help of a glass spreader. The plates were then allowed to dry for 15 mins. Whatman filter paper No. 1 paper discs of 6 mm diameter were loaded with plant sample (1mg/disc) and allowed to dry for some time. The discs were then placed over the surface of inoculated MHA media and left under the Laminar Air flow for 30 min at room temperature for diffusion. The plates are then kept for overnight incubation at 37°C. Chloramphenecol (10 µg per disc) and blank discs with DMSO were used as positive control and negative control respectively.

3.6. Determination of minimum inhibitory concentration (MIC)

Macro- broth dilution method was used to determine minimum inhibitory concentration (MIC). Serially diluted plant extracts were used according to the CLSI protocol (CLSI 2006). To get series of concentrations, the aqueous, methanol chloroform and petroleum ether extracts were diluted in sterile nutrient broth. 50µ of bacterial suspension was added to the broth dilutions. The test tubes with bacterial culture, media and different concentrations of plant extract were incubated along with two control tubes one containing media and bacterial culture and the other containing only nutrient media at 37°C for 18 hrs. The lowest concentration at which there was no visible bacterial growth was considered as MIC for each extract.
3.7. Determination of minimum bactericidal concentration (MBC)

The same procedure of broth dilution (Ericsson and Sherris 1971) as mentioned above was used to determine MBC. 50μl of microbial suspension from the tube that showed no visible growth after 18hrs of incubation was transferred to fresh media and again incubated for 18 hours at 37°C. The lowest concentration at which no visible growth of the freshly inoculated culture could be seen after overnight incubation was considered as MBC of each extract.

3.8. Determination of rate of bacterial killing

In order to study the rate of bacterial killing by the crude extract Eliopoulos amd Eliopoulos (1988) and Eliopoulos and Moellering (1996) protocol was followed with slight modifications. The extract with a concentration of 1xMIC was added to 10ml of Muller- Hinton broth. The control tube contained Muller – Hinton broth and test organism without extract. The tubes were incubated for 0h, 3h, 6h and 10h at 37°C at 120 rpm. 100 μl aliquot from each of the tube at specific time interval was plated in agar plates and incubated for 37°C. After incubation for 18h, the bacterial colonies were counted and compared with the count of the control bacterial culture.
3.9. Qualitative phytochemical screening

Qualitative phytochemical analysis was carried out to detect the presence of terpenoid, alkaloid, flavanoid, phenols, tannins and carbohydrates (Harborne 1998, Kokate 2000). Methanol, chloroform, petroleum ether and water extracts were freshly prepared for phytochemical analysis.

3.9.1. Test for alkaloids by Wagner’s method

5ml of 1% HCL was taken in tube and 2ml of each plant extracts was added into it. The mixture was allowed to stand on a steam bath for five minutes and then 1 ml of the filtrate was added to Wagner’s reagent. Brown/ reddish precipitation formation indicated the presence of alkaloids.

Wagner’s reagent was prepared by dissolving 2 g of iodine and 6 g of KI in 100 ml of water.

3.9.2. Test for flavonoid

To 0.5 ml of extract a few drops of sodium hydroxide was added. Occurrence of intense yellow colour which became colourless on addition of few drops of dilute H₂SO₄ indicated the presence of flavonoid.
3.9.3. Test for phenols by ferric chloride test

To 2ml of extracts 4-5 drops of ferric chloride solution was added. Presence of phenol was indicated by bluish balck colour formation.

3.9.4. Test for terpenoid by Salkowski test

3 ml of extract was added to a mixture of 2 ml of chloroform and 3 ml of concentrated H$_2$SO$_4$. Appearance of reddish brown colour at the interface indicated the presence of terpenoid.

3.9.5. Test for tannins

To 0.5 ml of each extracts, 5ml of chloroform and 1ml of acetic anhydride were added. Green colour formation after addition of sulphuric acid (1 ml) solution carefully to the solution along the wall sides of the tube was the positive indication of the presence of tannins.

3.9.6. Test for carbohydrates

Each extract was dissolved in water and then filtered. The filtrate was added to Benedict’s reagent and allowed to stand on a hot water bath. Appearance of orange red precipitate indicated the presence of carbohydrates.
Benedict’s reagent was prepared by dissolving 5 g of sodium citrate, 1.875 g of sodium carbonate and 3.125 g of potassium thiocyanate in 15.9 ml of hot distilled water. Then the solution was mixed with 2.5 ml of a solution of 8.38% (w/v) CuSO₄·5H₂O. Then 0.15 ml of 5% solution of potassium ferrocyanide was added to the solution and mixed thoroughly.

3.9.7. Test for steroids

A small amount of extracts were dissolved in chloroform and few drops of concentrated sulfuric acid were added to it followed by the addition of 2-3 drops of acetic anhydride. Formation of green colour indicated the presence of steroids.

3.10. GC-MS analysis

GC-MS analysis of the chloroform extract was performed using a Perkin–Elmer GC Clarus 600/680 system and a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with a Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) fused with a capillary column (60m x 250 μm). Helium gas (99.99%) was used as a carrier gas and an injection volume of 2 μl was employed (a split ratio of 50:1). The injector temperature was maintained at 200 °C, the ion-source temperature was 180 °C, the oven temperature was programmed from 50°C (isothermal for 2 min) with an increase of 5 °C/min to 300°C ending with a 8 min isothermal at 320 °C. Mass spectra were taken at 70 eV; scan fragments from 50 to 600 Da. The solvent delay was 14 min. Interpretation on
Mass-Spectrum GC-MS for identification of various metabolites present in the extracts was conducted using the database of National institute Standard and Technology (NIST).

3.11. Mechanism of action of plant extract on bacterial cells

3.11.1. Determination of oxidative stress on bacteria cells using nitroblue tetrazolium reduction (NBT) method (Banerjee et al. 2010).

0.1ml of Hanks' balanced salt solution (HBSS) was added to the bacterial suspensions of $10^5$ CFU/ml. Then the solution was incubated with plant extract for 5, 30, 60, 90 and 120 min time intervals, respectively at 37 °C. Each of the solutions were then treated with 0.5ml of nitroblue tetrazolium (1mg/ml). Each solution was then incubated at 37 °C for 30 min. To stop the reaction 0.1 M HCL was added to each of the solution and then cells were separated from the supernatant by centrifugation at 5000 rpm for 10 min. 0.4 ml of dimethyl sulfoxide (DMSO) was added to the pellet to extract out reduced NBT. To dilute the solution 0.8mL of HBSS was added. Formazan blue colour formed was measured using spectrophotometer at an absorption spectrum of 575 nm.

3.11.2. Preparation of cells for transmission electron microscope (TEM)

A high resolution transmission electron microscope (TEM, JEM 2100, Jeol) with an accelerating voltage of 200 KeV was used to study changes in bacterial cell morphology and cell envelope. Bacterial culture of cell density $10^5$ CFU/ml was
treated with the plant extract for 1 h at 37 °C. Untreated control cells were cultured in nutrient broth in absence of extract. Cell pellets obtained from the treated and control cell suspension were diluted with 0.1M sodium cacodylate buffer and mixed thoroughly with an equal volume of 2% glutaraldehyde and then left for 15 min at 4 °C. The pellet was obtained by centrifugation at 10000 rpm for 10 min and then the pellet was refixed with 2% glutaraldehyde for 1 h at 4 °C. 0.1M sodium cacodylate buffer was used to rinse the pellet and 2% osmium tetroxide (OsO₄) was used for post fixation. The dehydration of the samples were carried out with graded acetone series and embedding in pure embedding medium using beem capsules. Ultrathin sections were prepared in copper grids and allowed to dry. The sections on the grid were stained using a double staining technique involving uranyl acetate and lead-citrate staining.

3.11.3. Analysis of changes on plasmid DNA

For comparison of changes of DNA of extract treated and control bacterial cells DNA was isolated using Sambrook and Russell method (2001) with modification. The bacterial cells were treated with extract and incubated for 30 min, 60 min and 90 min. Then 1.5 ml of each culture was centrifuged for 30 seconds at 4°C. The supernatant was removed to get the pellet. The pellet was resuspended in 100µl of ice-cold alkaline lysis buffer (Solution I- 50mM glucose; 10mM EDTA 25mM Tris HCL, pH 8.0). Then the solution was vortexed vigorously. 200 µl of freshly prepared solution II (0.2 N NaOH, freshly diluted from a 10 N stock; 1% (w/v) SDS) was added and the solution was mixed by inverting the microcentrifuge tube rapidly five
times. The solution III (3 M KoAC/Acetic Acid) was added and the tube was inverted several times. The supernatant obtained after centrifugation was transferred to a fresh tube and 1µl (10mg/ml) RNAse was added to each sample and incubated at 55°C for 45-60 min. Equal volume of phenol:chloroform was added and the mixture was vortexed and centrifuged at a maximum speed at 4°C. The upper layer was transferred to a fresh tube and precipitated by ethanol. The DNA was collected by centrifugation and the supernatant was removed. The dried DNA pellet was dissolved in 50 µl of TE (pH 8.0) and vortexed gently. The DNA was stored at 20°C.

For analysis of DNA band, 4µl of each sample solution was mixed with 2 µl of 6X loading dye and subjected to run at 0.8% agarose gel electrophoresis from Bio-Rad laboratories. Ethidium bromide was used to stain the gel. The gel was visualized using a gel documentation system.

3.12. Fractionation of crude extract

The most active crude plant extract was separated into individual fractions by column chromatography. Each fraction was then subjected to antibacterial assay. The active fraction was then further separated using thin layer chromatography (TLC).

3.12.1. Column chromatography

Column chromatography, the most common method used by the researchers for separation and purification of natural products includes two phases, one stationary phase made of silica, alumina, magnesia etc. and the other is liquid phase. The adsorbent is made
into slurry by dissolving in a suitable solvent placed in glass column plugged at the bottom. The extract or the mixture to be separated is dissolved in a suitable solvent and then poured at the top of the column and allowed to pass through the column. Different components of the extracts get adsorbed at different region of the column depending on their adsorption ability. The components are eluted by adding solvents of suitable polarity at the top.

3.12.1.1. Column packing

A glass column (Borosil 300X18 mm diameter) with PTFE stopcocks and sintered disc was used for column chromatography. A ring stand was used to clamp the column and one fourth of the column was filled with initial eluent (hexane). Slurry of activated silica gel (60-120 mesh, Merck Germany) was prepared by pouring hexane into a beaker containing dry silica. Care was taken to make the slurry free from air bubble. Using a Pasteur pipette the slurry was added to the column. The stopcock was kept open to drain the excess solvent and fresh hexane was added to the silica gel. This process helped the silica gel to settle down. The process was repeated several times. A rubber stopper was used to tap the column on the sides which helped the silica gel to settle uniformly.

3.12.1.2. Sample loading

The dry crude extract was dissolved in hexane and was added to the silica layer with help of a pipette carefully.
3.12.1.3. Sample elution

Elution of the sample was done initially with 100% hexane and then sequential elution with solvent system of increasing polarity- hexane: DCM (dichloromethane) in the ratio of 1:1, hexane: DCM (2:8), 100% DCM, 100% chloroform, 100% ethyl acetate, ethyl acetate: methanol (1:1), methanol (100%) was done. Different fractions were eluted in 15ml vial each and then solvent was allowed to dry.

3.12.1.4 Analyzing the fractions

Each of the fractions eluted were monitored using comparative thin layer chromatography (Co-TLC). Similar fractions were combined without affecting the purity. Each fraction was then separated into sub-fractions using TLC. The solvent system in which more bands were seen for the bioactive fraction was used for elution of the bands in preparative TLC. Merck TLC plates (60G F254 20X20cm) were used for band elution.
Ethnomedicinal Survey

Plant collection

Extraction

Bioassay of crude extract

Fractionation

Bioassay of fractions

Sub- Fractionation of bioactive fractions

Pure Active Compound

Structure Analysis

Figure 3.2. Flow chart showing the process used for isolation of antimicrobial compound
3.13. Overview of plants with antibacterial activity

In the present study different ethnomedicinal plants collected were screened for antibacterial activity and the following two potent plant species were selected for further analysis.

3.13.1 *Litsea salicifolia* (J. Roxburgh ex Nees) Hook. f ( Voucher No. GUBH <17869>)

*L. salicifolia* belonging to the family Lauraceae is an evergreen, tall (10m) tree. Leaves of these plants are elliptic to lanceolate and the colour of the leaves is variable from green to pale brown to darker above when dry. Flowers are unisexual. *Litsea salicifolia* (Roxb. ex Nees) Hook. f is commonly known as 'Dighloti' in Assam. *L. salicifolia* leaves are used by Assamese people in Bohag Bihu, an Assamese cultural festival to clean their cattle, because leaves of dighloti is believed to have medicinal properties.

**Systematic position:**

Kingdom: plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Laurales

Family: Lauraceae

Genus: *Litsea*

Species: *L. salicifolia*
3.13.2. *Mucuna pruriens* (L.) DC. (Voucher no. GUBH <18164>)

*M. Pruriens*, also known as velvet bean is an annual tropical leguminous plant. Young plants are covered with hairs. Leaves of *M. Pruriens* are tripinnate, ovate and rhombus in shape. Young leaves are covered with hairs. The flowers remain in clusters and are usually white or purple in colour. Cluster of seedpods are also produced by the plant. It is being used traditionally as a source of medicine in different countries. Pods are used for human consumption in many places like Asia, America, Africa and Pacific islands. Stinging hairs of the plant causes itching sensation. In Assam *M. Pruriens* is known as ‘Bandar Kekowa’.

**Systematic position:**

Kingdom: plantae  
Division: Magnoliophyta  
Class: Magnoliopsida  
Order: Fabales  
Family: Fabaceae  
Genus: *Mucuna*  
Species: *M. pruriens*
3.14. Statistical analysis

All experiments done in triplicate are represented as mean ± SD (standard deviation). T-test was used for comparison of two groups and Tukey–Kramer multiple comparison tests was used for comparison between more than two groups. Values at P<0.05 were considered statistically significant.
Plate 1. A. *Litsea salicifolia* (J. Roxburgh ex Nees) Hook. f, B. *Mucuna pruriens* (L.)

Plate 2. Bacterial cultures in agar plates (A) E. coli 443 (B) *Pseudomonas aeruginosa* 424 and (C) *Staphylococcus aureus* 96 and (D) *Streptococcus pneumonia* 655